

Recent Advances in the Detection of Allergens in Foods

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Abstract

Food allergy is a public health issue that has significantly increased worldwide in the past decade affecting consumers' quality of life and making increasing demands on health service resources. Despite recent advances in many areas of diagnosis and treatment, our general knowledge of the basic mechanisms of the disease remained limited, i.e., not at pace with the exponential number of new cases and the explosion of the new technologies. For sensitized individuals, the only effective way to prevent allergic reactions is the strict avoidance of the offending food. For this reason, a number of regulatory bodies in several countries have recognized the importance of providing information about the presence of food allergens by enacting laws, regulations, or standards for food labeling of "priority allergens." This has resulted in the need for the development of analytical methods for protection of food-allergic consumers that should be among others highly specific, sensitive, and not influenced by the presence of the food matrix components. Several analytical approaches target either the allergen itself or a corresponding allergen marker such as peptide fragment or gene segment and have been used in the detection and quantification of allergens in food products. In this short review, some of the conventional and new methods for the detection of allergens in food are listed and briefly discussed.

Key words Food allergy, Allergens, Sensitization, Analytical methods, ELISA, Mass spectrometry

1 Introduction

Food allergy is a public health issue that has significantly increased worldwide in the past decade, affecting consumers' quality of life and making increasing demands on health service resources [1–3]. The statistics vary but according to the EAACI 2015 report approximately 17 million people in Europe are suffering from food-related allergies and critical hospital admission for children has increased by sevenfolds within the past 10 years. Food allergy affects around 2–4% of the population peaking at 6–8% at 1 year of age and declining gradually to 2–4% in older children and adults [4–6].

This decline reflects the fact that many infants grow out of their food allergies as they age. The rate and probability of resolution varies significantly among specific foods. Frequently,

egg, milk, wheat, and soy allergies resolve relatively early in life, whereas allergies to peanuts, tree nuts, sesame seeds, fish, crustacean shellfish, and buckwheat are likely to persist. Truthfully, to this day, it remains unclear why some children or adults achieve natural tolerance and others do not. The estimated rates of resolution also vary among studies for any specific food and only contributing resolution factors such as sensitization to multiple food allergens, high allergen-specific IgE antibody levels, a history of anaphylaxis, atopic dermatitis, and particular IgE-binding patterns for food allergies.

The degree and type of childhood food allergy, in particular, is an evolving public health problem that appears to have risen rapidly in industrialized countries. However, although it is estimated that 10–15% of children report symptoms of food allergy, the actual prevalence of IgE-mediated food allergies (i.e., symptoms of food allergy in the context of a positive skin prick test) is reported to be much lower at approximately 6–8% in children less than 3 years old [4, 5]. By contrast, not much is known about the prevalence of non-IgE-mediated food allergies, although both eosinophilic esophagitis and celiac disease have been documented to be increasing. Despite the increasing number of studies mounted to investigate the rise of both allergic diseases in general and food allergy in particular, the causes behind the putative epidemic of food allergy remain elusive. In general terms it is estimated that about a quarter of the population will have an adverse reaction to food (of which food allergy is just one type) during their lifetime, most of which will occur during infancy and early childhood.

Regarding the type of food involved and despite the geographical variations attributed to differences in environmental or individual factors, it is observed that 75% of allergic reactions among children are linked to a limited number of foods, which include egg, peanut, cow's milk, fish, and various nuts. Conversely in adults, fruits of the latex group (e.g., kiwi, banana), fruits of the Rosaceae family (e.g., apples, pears, prunes), vegetables of the Apiaceae family (e.g., carrot, celery), and various nuts and peanuts are responsible for 50% of allergic reactions [7–9].

1.1 Clinical Symptoms

IgE-mediated allergic reactions to food are represented by well-defined clinical features. These allergic reactions may manifest as a variety of signs and symptoms that can involve the digestive, respiratory, cardiovascular, or cutaneous organ systems [10]. Reaction severity varies from mild (skin rashes, itchy nose and eyes, sneezing, wheezing, coughing, itchy lips and mouth, nausea, cramp, bloating, vomiting, and diarrhea) to severe (such as systemic shock). Reactions involving multiple organ systems are generally called anaphylaxis. Fortunately, most allergic reactions to food are relatively mild but in very rare cases allergic reactions can be fatal. Reaction severity depends on the age, sensitivity of the individual, the level of exposure (amount

of food consumed), and modifying factors such as the presence of other conditions (e.g., uncontrolled asthma) or the use of medications such as steroids or antihistamines. Clinical studies have shown that the range of sensitivity in the allergic population can encompass many orders of magnitude [11, 12]. This means that the same exposure might result in a mild reaction in one individual, a severe reaction in another, and death in a third.

1.2 What Defines an Allergen?

In general terms, food allergy can be defined as an immune-mediated sensitivity to food proteins [10]. It is essentially “immunity gone wrong” where normally harmless substances (food allergens) are perceived as a threat and acted upon by the body’s immunological defenses. In a type I hypersensitivity reaction the adaptive immune system responds by producing IgE antibodies against the allergens. It is a two-step process where the capacity to respond through an allergic reaction when exposed to the particular allergen must be first established. This induces the immune system to generate specific IgE antibodies against the allergen in a so-called induction phase, or sensitization. Once sensitized to a particular allergen, the individual may develop a symptomatic allergic reaction when exposed again to the allergen in question in a provocation or later triggering phase [13].

Interestingly what defines a major allergen is still largely unknown. More importantly, the intrinsic factors that differentiate a common everyday food protein from a fatal allergen to a particular group of patients remain largely unknown. Despite attempts by a large number of groups to define the basic intrinsic characteristics of an allergenic protein, only two mechanisms, both described in the *Dermatophagoides pteronyssinus* (house dust mite), are currently known: a protease-dependent (Der p 1) and a protease-independent one (Der p 2). In the former, Der p 1 protease activity has been shown to cleave CD23, CD25, and CD40, inhibiting IL-12 secretion with the possible activation of the PAR family of G coupled proteins in the process [14]. In the latter mechanism, it has been elegantly shown that Der p 2 structurally mimics MD2, a component of the TLR4 complex that can coopt other signaling components to activate its target cells [15]. Hence, the involvement of complex lipids and Toll receptors has been clearly demonstrated. However, as most of the well-characterized allergens are not proteases and it is unlikely that MD2 homology could be a general mechanism for allergenicity these suggest that other mechanisms certainly exist [16].

This means that current understanding of the molecular mechanisms underlying the development of IgE responses is incomplete and as a result any risk assessment regarding allergenicity has to rely on historic evidences. Factors such as the age when the initial sensitization occurred, genetic susceptibility, environmental factors including geographic distribution, exposure route, type of food,

food matrixes, adjuvants among many others should be considered. As a consequence of this complexity decision-tree approaches have been proposed to facilitate the allergenicity assessment (ILSI and its revised version proposed by a joint FAO/WHO committee) [17]. The criticism and limitations of the different approaches currently used in the assessment of allergenicity are beyond the scope of this article; however, it should be noted that even the now ethically challenging and expensive animal experiments have shown not to mimic the ability of humans to become sensitized, hence alternative methodologies are sought.

1.3 Processing and Threshold

Cooking and food processing as a whole can affect the ability of the food components to trigger a reaction in an unpredictable manner. Nevertheless, information on minimal eliciting doses for various food allergens has emerged in recent years. The minimum allergen concentration to trigger an allergic reaction or threshold for hazelnut, for example, is about 30 μg of the nut [18] with values of 0.07 μg for milk, 0.003 μg for egg, 0.5 μg for peanut, and 0.3 mg for soybean [19]. However, due to the large differences in threshold values between individuals, a universal figure for the maximum concentration of an allergen within a food has yet not been determined since trace amounts of allergens can induce severe and even fatal reactions in sensitive patients. While there is interest by several stakeholder groups (i.e., food industry, regulatory agencies, allergic consumers, and clinicians) on threshold information, many countries' regulatory agencies have not yet got them implemented [12]. As there is so much uncertainty regarding each individual, any exposure to protein from a food allergen is considered to be of public health significance; hence, the food industry is tasked with essentially a zero threshold level of allergenic residue. This is operationally impossible given the complexity of manufacturing facilities and the numerous routes of allergen contamination and cross contact that can occur throughout the supply chain.

1.4 Legislation

The availability of information about the presence of allergenic food ingredients that are intentionally used in a food product has improved significantly in the last 15 years. Initially, a list of priority allergens was developed by the Codex Alimentarius 1999 [20] and used as a starting point by the European Commission and other regulatory bodies [21]. Although more than 180 allergenic food proteins have been described to date, in practical terms and based on prevalence, severity, and potency, a relatively limited number of food components have been identified as allergens [21, 22]. The European Regulation (EU) 1169/2011/EC (OJEU, 2011) provides a list of 14 groups of allergenic food components that manufacturers are required to declare on their labels if the ingredients are present regardless of their quantity. The list includes gluten-containing cereals, crustaceans, eggs, fish, peanuts, soybeans, milk, nuts (almond,

hazelnut, walnut, cashew, pecan nut, Brazil nut, pistachio nut and macadamia nut), celery, mustard, sesame seeds, lupine, and molluscs, and products thereof. In addition, sulfur dioxide/sulfites are also required to be labeled at levels >10 ppm as they can trigger asthma attacks in those who are sensitive to this ingredient, and have also been associated with anaphylaxis [23].

While declaration of ingredients derived from allergenic sources used as direct ingredients or processing aids has helped to provide allergic consumers with more transparent allergen information, labeling laws do not address the potential risk involved with undeclared or hidden allergens. Hidden allergens may be present in the food products as a result of their unintentional or undeclared presence in the raw materials used, and also due to cross-contamination produced in shared equipment (e.g., use of common equipment, improper production scheduling, or improper use of rework material), to the mixture of ingredients at the supply chain level, or to a fraudulent adulteration. Cross-contact can occasionally occur despite the food manufacturer's best efforts to remove the allergenic residue [24].

2 Introduction to Techniques

Several analytical approaches have been developed for the detection and quantification of allergens in food products. These techniques target either the protein allergen itself or a corresponding allergen marker (peptide fragment or gene segment). Due to the diverse nature of food allergens and the complexity of the respective food matrices, the choice of detection methods can be challenging at times. With the recent advances in molecular biology, biochip technology, mathematical modeling among others and access to large volume of information on genomics, genomic-associated studies, cell culture, cell engineering, microbiome, and many of the new omics, the whole diagnostic, therapeutic, and basic immunology area of food allergy is undergoing a large transformation. At the protein level allergens are mainly determined by antibody-based enzyme-linked immunosorbent assays (ELISA), protein biosensors, and mass spectrometry (MS) methods [25–27]. However, due to their thermo/mechanical and chemical instability protein-based methods are only applicable to raw or slightly processed food components [28]. In contrast, the higher stability of nucleic acids makes it possible to apply DNA-based methods to both raw and highly processed food. Most DNA-based methods involve amplification of the target sequence by PCR or real-time PCR [29, 30]. Analytical methods used for the detection of residues of allergenic foods must not only be highly specific, sensitive, and rugged enough to be applicable in food matrices but also reliable. Moreover, the testing methods should detect levels of

contaminants at concentrations in low parts per million range (ppm). Within this context in the next paragraphs a brief description of the common techniques applied in the detection of allergens in food products is listed and discussed.

2.1 Protein-Based Techniques

2.1.1 Immunoassays

Immunoassays are a collection of techniques that utilize specific antibodies raised against a specific antigen. The antibodies normally employed in immunoassays are mainly of two categories: polyclonal, when antiserum comprises immunoglobulins secreted by different B cell clones that recognize different epitopes, or monoclonal, if the immunoglobulins are secreted by clones of a single hybridized B cell and therefore react against a single epitope. Polyclonal and monoclonal antibodies are principally raised in animals, and mainly fall into the IgG antibody class, although when produced in egg yolk they are designated IgY [11, 31, 32]. A different strategy aimed to overcome the downsides of traditional antibodies (variations among different batches, use of live animals, etc.) entails their replacement by recombinant antibodies, such as scFv and Fab as described in Chap. 9 in this volume. Recombinant antibody technology makes it possible to create libraries of antibody fragments derived from immune cells or synthetic libraries and to employ phage display technology for panning antibody fragments with the desirable affinity [33]. Antibodies can also be replaced by oligonucleotide aptamers, ligands selected from combinatorial libraries or peptide molecules that bind their targets with high affinity [34, 35]. Due to their high sensitivity and specificity, immunoassays are frequently used analytical methods for the detection of food allergens, providing an accurate analysis of the test sample. Among immunoassay methods, enzyme-linked immunosorbent assay (ELISA) and lateral-flow devices (LFD) are the most commonly used formats.

ELISA

The enzyme-linked immunosorbent assay (ELISA) is the most widespread technique employed in industries and official food control agencies for the detection of allergenic ingredients in food products. It is reasonably quick, reliable, user-friendly and does not require special equipment allowing the simultaneous analysis of several samples [32, 36–38]. Several companies have developed commercial test kits that have become very popular for the food industry (Table 1). As described in Chap. 8 one of the main issues with ELISAs relates to the efficiency of the extraction methods. It has been demonstrated that certain food matrix components can inhibit the extraction of proteins or interfere on the detection of the analytes. To optimize the extractability of allergens, the use of extraction additives such as fish gelatin, skim milk powder, bovine serum albumin, is advisable [39]. ELISA methods employed in food industry are usually presented in a sandwich format. In this design, a capture antibody aimed to retain the antigens present in

the sample is coating the plate surface. Captured antigens are later detected by a secondary antibody, which can be conjugated to an enzyme converting the substrate in a detectable product. However, when detecting small-size proteins, the preferred format is the competitive ELISA assay format where antigen is coated to a plate surface and the antibody is incubated with a diluted sample extract (inhibitor) before being added to the plate [31, 40]. ELISA results can be affected by cross-reactivity problems; thus positive ELISA results might require a confirmatory analysis by a non-immunological technique to corroborate data and to improve detection specificity [41].

Lateral Flow Devices (LFD) LFD are fast and easy-to-use immunoassays test employed routinely in food industries (Table 2). They consist of a membrane strip with a sample pad on which a liquid sample containing antibodies or antigens is applied. Their major advantage is that they do not require specific instrumentation, but they solely determine the presence or absence of the target, not being suited to quantification of the amount of analyte present in the sample [31, 42]. Similar to ELISA lateral LFD are presented in two different formats: sandwich and competitive. Sandwich LFD is the most widespread design in food allergen testing where the sample is loaded into a membrane strip containing the specific antibodies against the target that remain unbound to the membrane. The detection

Table 2
Commercial ELISA kits for detection of food allergens

LFD	Company name			
	Crystal Chem	R-biofarm	Romer Labs	Elution technologies
Targets	Casein Gliadin/gluten Egg	Almond	Almond	Almond
		Brazil nut	β-lactoglobulin	Cashew
		Cashew	Brazil nut	Coconut
		Coconut	Casein	Egg
		Crustaceans	Cashew/pistachio	Fish
		Egg	Coconut	Gluten
		Lysozyme	Crustaceans	Milk
		Gliadin/gluten	Egg	Peanut
		Hazelnut	Hazelnut	Pecan
		Macadamia nut	Lupin	Pistachio
		Milk	Macadamia	Soy
		Mustard	Mustard	Walnut
		Peanut	Peanut	
		Pistachio	Sesame	
		Sesame	Soy	
		Walnut	Milk	
			Walnut	

antibodies are labeled with colored particles (latex or gold nanoparticles), enzymes, or fluorescent molecules. In competitive LFD format, the analyte is attached to an antibody or protein that is labeled with latex or a colloidal metal (detection reagent). After loading the sample, the detection reagent and the sample containing the target molecule will wick onto the membrane; they will reach the test line region containing the capture antibodies (immobilized antibodies against the target analyte), which will capture some of the analytes and some of the detection reagents. The more analyte present in the sample (that is unlabeled), the more will compete with the limited amount of labeled particles comprising the detection reagent. The test line will appear as a colored band in samples not containing target molecules. The control line will appear as a band as the labeled antibody is captured by an antibody that is specific for the labeled antibody.

2.1.2 Protein Biosensors

Biosensors provide a novel approach for the detection of allergenic ingredients in food products [43]. They can be small portable real-time devices that may be used for on-site analyses. By the immobilization of a sensor onto a chip surface, whether it is an antibody recognizing the allergenic protein, a specific DNA fragment encoding for an allergen recognizing a complementary strand or an aptamer selected to bind the specific allergen, the binding interaction between the two can be measured quantitatively. Biosensors consist of a sensing component of biological origin for selective detection of target, and a receptor-transducer analytical device that transforms the interaction into a measurable chemical-physical signal proportional to the target concentration [44]. According to the physical change accompanying the reaction, biosensors can be classified into optical and electrochemical types.

Optical biosensors detect changes in light absorption when the analyte is recognized by the receptor, or differences in light output as a result of a luminescent process. Many optical biosensors are based on surface-plasmon resonance (SPR), which exploits the changes in the refractive index close to a sensor surface. SPR biosensors have been applied in several food allergen detection methods. Pollet et al. [26] reported a fiber optic SPR biosensor that employed paramagnetic nanoparticles coupled to Ara h1 antibodies that allowed amplifying SPR signals when detecting Ara h1 peanut allergen. Billakanti and coworkers [45] presented a SPR technology for the simultaneous quantitative determination of five whey proteins employing antibodies immobilized on a GLC biosensor chip (Bio-Rad Laboratories). The instrument employed was a ProteOn XPR36 protein interaction array system (Bio-Rad Laboratories, CA, USA). Rebe Raz et al. [46] employed an angle scanning imaging SPR in combination with antibody array to rapidly quantify 13 major food allergens in foodstuffs. Studies that employ optical biosensors different to SPR are also described in the

literature. Yman et al. [47] developed an optical biosensor for both direct and sandwich immunoassays detection of proteins from milk, egg, hazelnut, peanut, shellfish, and sesame in food samples. Godoy-Navajas and coworkers [48] developed a long-wavelength fluoroimmunoassay to determine the presence of soy proteins in food samples employing Nile blue-doped silica nanoparticles. Furthermore, an assay for the simultaneous identification of eight food allergens (soybean, wheat, peanut, cashew, shrimp, fish, beef, and chicken) by employing silicon-based optical thin-film biosensor chip was reported by Wang et al. [49]. As the method allowed appreciating the change in color by the naked eye, there was not need of sophisticated equipment.

Electrochemical biosensors provide analytical information using a biological-recognition element (biochemical receptor), which is retained in direct spatial contact with an electrochemical transduction element. Electrochemical biosensors can be classified into potentiometric, amperometric, voltammetric, and impedance types, according to the mode of physico-chemical signal transduction. Cao et al. [50] determined the presence of casein through differential pulse voltammetry employing an immunosensor based on anti-casein antibody immobilized on a composite film based on gold nanoparticles and poly (L-Arginine)/multi-walled carbon nanotubes. Eissa and coworkers [51] developed a label-free voltammetric immunosensor to detect β -lactoglobulin employing graphene-modified screen-printed electrodes. Yang and coworkers [52] reported a concanavalin A-based electrochemical biosensor for the detection of chicken ovomucoid based on self-assembled ZnO quantum dots. Jiang and coworkers [53] developed a cell-based electrochemical biosensor to quantify Pen a1 shrimp allergen. To that end, mast cells were immobilized on L-cysteine/gold nanoparticle-modified gold electrodes. Immobilizing a monoclonal antibody on a gold electrode, Huang and coworkers [54] developed an impedance biosensor aimed to detect peanut allergenic protein Ara h1. They also described an electrochemical biosensor in which gold electrode is replaced by a degenerate Si electrode [55]. Singh and coworkers [56] reported a nanopore biosensor to detect peanut protein Ara h1, which is constructed by immobilizing an antibody within gold-coated pores of commercial nanoporous polycarbonate membranes. As the pores are obscured by the antibody-antigen complexes, electrolyte conductivity within the membrane decreases.

Electromechanical biosensors or quartz crystal microbalance (QCM) biosensors, the biological-recognition event generates a mass change of the sensing layer, giving rise to a change in the resonant frequency of the microbalance. The application commonly requires the immobilization of antibodies or DNA fragments on the transducer surface. Xiulan et al. [57] developed a QCM-based immunosensor aimed to determine the presence of

shrimp allergen in food. Antibodies against shrimp were attached onto the crystal surface via a nanogold-modified composite film. The presence of the antigen bound to the antibody will induce a change in the frequency proportional to the concentration of the binding antigen. Chu and coworkers [58] described a QCM biosensor for the detection of gliadin in foods. The incorporation of gold nanoparticles increased by 48% the shift in the frequency of the biosensor in the detection of 2 ppm gliadin.

2.1.3 LC-Mass Spectrometry Techniques

Mass spectrometry (MS) is an analytical technique employed in food allergy research to obtain structural and functional information about food allergens, and it also serves as a non-immunological method to detect the presence of allergens in foodstuff. MS serves as a non-immunological method for fast, accurate, and sensitive analysis of traces of food allergens. The term “allergenomics” has been coined to define the application of proteomic technologies to the analysis of food allergens [59, 60]. The most widely employed soft-ionization techniques in use are matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). MS is usually coupled with separation techniques as 2D PAGE, or liquid chromatography (LC), which enables the analysis of proteins or its peptides with high sensitivity and specificity. 2D PAGE allows the simultaneous separation of a large number of proteins according to their isoelectric point and their molecular weight. Protein spots are excised from the gel, digested with trypsin, and identified by MS or tandem mass spectrometry (MS/MS). MS identifies the protein by peptide mass fingerprint, providing evidence for the most probable identity of the protein, whereas MS/MS analysis offers structural information of the molecule (amino acid sequence of detected peptides), making the search highly specific and discriminating [32, 61, 62]. This methodology is known as the bottom-up approach. An alternative strategy, known as top-down proteomics implies the introduction of the intact protein into the mass spectrometer to measure the intact and fragment ions masses. This approach allows the full characterization of proteoforms (highly related protein molecules arising from all combinatorial sources of variation giving rise to products arising from a single gene). The latter approach is nowadays possible due to the high mass accuracy of new high-resolution mass spectrometers [63–65]. On the other hand, shotgun proteomics employs liquid chromatography (LC) coupled to MS analysis as an alternative to electrophoretic methods to separate proteins. In such an approach, the entire protein extract is digested by trypsin before being separated by LC (usually reverse-phase chromatography (RP))-ESI (electrospray ionization)-MS/MS. Liquid-phase separation method has higher analytical dynamic range, thus allowing the detection of low-abundance components—which becomes very useful when detecting traces of food allergens—but its resolution is limited compared to 2D-PAGE [66–68].

Therefore, to separate highly complex peptide mixtures, multidimensional identification (MultiID) technology is the preferred option, as it offers good resolution and peak capacity. The most common combination is strong cation-exchange resin coupled with reverse-phase LC (RPLC), but other formats employed included size-exclusion chromatography (SEC) with RPLC, and RPLC with capillary electrophoresis (CE). Recent advances in MultiID include peptide separation by ultra-high pressure LC (UHPLC) and anion- and-cation mixed-bed ion-exchange techniques [69, 70].

Capillary-liquid chromatography (CLC) and nano-LC are miniaturized techniques with great potential in the analytical field. Nano-LC employs capillaries with an internal diameter (ID) lesser than 100 μm and flow rates of 50–800 nL/min, whereas in CLC capillaries with an I.D. of 100–320 μm and flow rates of 1–100 $\mu\text{L}/\text{min}$ are used. The reduction of column ID translates in shorter times of analysis, lower consumption of mobile phase, and easier coupling with MS because of the lower flow rates when comparing it with conventional LC. It also increases mass sensitivity due to the reduction of the mobile-phase flow responsible for the decrease in sample dilution. However, low I.D capillaries require a reduction in extra-column effects that can affect the efficiency of the technique. Considering the injected volumes, the sensitivity of the technique can be questionable for practical purposes. To solve this problem, it has been proposed the use of more sensitive detectors and/or applying sample pre-concentration (off-and on-column) [71].

When working with MS techniques, it must be taken into account that the quality of the protein identification will depend upon the quality of the mass spectrometry data, the accuracy of the database, and the power of the search algorithms and software used. Some examples of MS application in food allergen detection are stated below: Weber and coworkers [72] employed a CLC-Q-TOF MS/MS to detect αS1 -casein in spiked foods, employing as a marker the casein peptide m/z 634.3. Monaci and coworkers [69] developed a CLC-ESI-MS/MS method for the detection of α and β -casein derived peptides in fined white wine, in which four specific peptide ions (m/z 742.2, 729.2, 634.2, and 692.6) could be identified by CLC-ESI-MS/MS analysis followed by bioinformatics search. They described a LOD 50 $\mu\text{g}/\text{mL}$ for caseinate added to wine, when the m/z 742.2 ion is used as a caseinate marker. In Monaci et al. [73], they described the detection of milk protein and caseinates in two commodities (cookies and white wine) using a RP-LC separation coupled to an ion-trap mass spectrophotometer. Heick and coworkers [74] developed a LC-MS/MS method for the simultaneous detection of seven allergenic foods (milk, egg, soy, peanut, hazelnut, walnut, and almond) in processed and unprocessed samples. The influence of the baking process was assessed, and the results were compared to those obtained with commercially available ELISA kits. The MS method showed supe-

rior detection capability for the processed samples when comparing its results to those obtained with commercially available ELISA kits, with the advantage of its capability of multiplexing.

In order to identify peptide markers of the allergenic proteins of peanut taking their thermal stability into account, Chassaigne and coworkers [75] described a CLC method combined with Q-TOF MS/MS. They defined two specific markers for the allergenic protein Ara h 1 (VLEENAGGEQEER (m/z 786.88, charge 2+)) and DLAFPGSGEQVEK (m/z 688.85, charge 2+), one for the Ara h 2 ((RQQWELQGDR (m/z 439.23, charge 3+)) and two for the Ara h 3 (peptides, SPDIYNPQAGSLK (m/z 695.35, charge 2+) and SQSENFYVAFK (m/z 724.84, charge 2+). Pedreschi et al. [67] employed shotgun proteomics to detect the presence of the proteotypic Ara h 3/4 marker peptides AHVQVVDNNGNR (m/z 432.5, 3+) and SPDIYNPQAGSLK (m/z 695.4, 2+) in baked cookies, employing as the representative food allergen source the standard IRMM-481 f. Mattarozzi et al. [66] described a sensitive and selective LC-ESI-MS/MS method to detect and quantify lupine's allergens (conglutins) in pasta and biscuits.

Undoubtedly, MS methods are a powerful tool to be employed in food allergen detection, overcoming the problems encountered with immunological techniques due to lack of detection of allergens after food-processing treatments that cause structural changes of proteins. However, their application requires the use of expensive equipment with high maintenance costs, and it needs to be operated by highly trained personnel. Therefore, at the present, MS methods cannot be employed as a routine analysis to trace the presence of food allergens, but can be used as an auxiliary method to confirm the results obtained with less expensive assays platforms routinely employed in food industry.

2.2 DNA-based techniques

Methodologies based on genetics and molecular biology have become an interesting approach for tracking down the presence of trace amounts of allergens at any stage along the food supply chain [76, 77]. DNA-based technology is a relatively new approach for the detection of food allergens, a field traditionally dominated by immunoassays. DNA assays have been used for food authentication, including speciation, and for the detection of products of agricultural biotechnology to ensure label accuracy and compliance with regulations. DNA methods are indirect methods to detect food allergens as they do not detect the allergenic protein itself, but the DNA sequences in the allergenic food. Usually, DNA methods are based on the amplification of specific DNA fragments either by means of end point PCR, which offers a qualitative result (i.e., it detects the presence of a specific DNA sequence), or by real-time PCR, which gives a quantitative account. In this sense, the polymerase chain reaction (PCR), together with real-time PCR, can be a valuable tool to indicate the presence or absence of a particular

allergen in food (below a concentration of 10 ppm). These DNA-based analytical methods are rapid, sensitive, and provide specific detection of the targeted DNA. The targeted DNA sequences are not necessarily located in the genes encoding for the allergenic protein, and thus the analysis detects genomic DNA of the offending ingredient, but does not necessarily indicate the presence of the protein that is responsible for the allergic response [78].

Advantages of DNA-based methods over protein-based methodologies rely in that the target DNA is efficiently extracted from raw and cooked products and is not affected by the heating process, because DNA typically remains intact after being exposed to the cooking temperatures of most foods. Although some components from food matrix may interfere with the PCR or real-time PCR assay, and therefore lower the amplification efficiency, PCR inhibitors can be removed during DNA extraction and purification steps with the help of suitable DNA clean-up procedures [79]. Quantification of allergens, either by immunological or by DNA-based methods, is a challenging problem, mainly due to the unavailability of certified reference materials (CRMs). Certified reference materials (CRMs) are materials or substances whose property values are sufficiently homogenous and well established to be used for method development and validation, or for proof of method performance, and possess certificate and traceable assigned values (to an international system, method or artifact). The use of these CRMs has in general contributed to facilitate compliance with food labeling laws and to improve the accuracy of analytical methods.

However, there is a current lack of reference materials for the development of allergen detection methods, which has prompted the scientific community to proactively working at this regard. Up to date, only a CRM has been developed for the detection of food allergens, and only for peanuts (Reference samples supplied by the Institute for Reference Materials and Measurements of the European Commission's Joint Research Centre, Belgium). Several research networks are currently involved in the production of food allergen reference materials, like egg powder and skimmed milk powder provided by NIST [28]. Even though reference materials are commercially available for the most important food allergens, the results obtained may not be comparable as these are not certified.

2.2.1 PCR and Real-Time PCR

A high number of PCR assays have been published for the detection of allergen residues in raw ingredients, and processed products (Table 3). PCR-based methods to detect DNA from an allergenic source in a food product consist of three steps:

1. DNA extraction and purification.
2. Amplification of specific DNA sequence(s).
3. Detection of the amplified DNA.

Table 3

Published DNA-based methods for detection of food allergens [RT-PCR (real-time PCR) NR (not reported)]

Allergen	Method	Target gene	LOD	Food matrices tested	Reference
Peanut	PCR	<i>trnL (chloroplast tRNA)</i>	NR	Peanut	[117]
		<i>Peanut agglutinin precursor</i>	10 ppm peanut DNA in wheat samples	Tree nuts, soybean, potato, corn, rice, wheat, meats, fish, commercial foods	[118]
	RT-PCR	<i>Arab2</i>	2 ppm peanut in biscuit	Tree nuts, meat, fish, seeds, biscuits, chocolate, cake, pastry, sausage	[119]
		<i>Arab2</i>	<10 ppm peanut	Cereal bars, chocolates, cookies, pudding	[120]
		<i>Arab3</i>	<2.5 pg peanut DNA or 10 ppm peanut in food	Cereals, legumes (soy, lupine), tree nuts, meat, seeds, cookies	[121]
Almond	RT-PCR	<i>Arab2 (1) ITS (2)</i>	10 ppm (1)/0.1 ppm (2)	Tree nuts, seeds, meat, plants, commercial food products	[89]
		<i>Pru1</i>	NR	Cookies	[91]
		<i>ITS1</i>	0.1 ppm	Commercial food products	[122]
Hazelnut	PCR-ELISA	<i>Cor a 1.0401</i>	<10 ppm hazelnut	Food and food ingredients	[123]
	PCR-HPLC	<i>Cor a1</i>	5 pg hazelnut DNA	Breakfast cereals, chocolate, biscuits, snacks	[124]
		<i>Cor a1</i>	10 ppm	Chocolates, snacks, muesli	[125]
	RT-PCR	<i>Nad1</i>	10 ppm in chocolate	Chocolate	[126]
		<i>Cor a 1.04</i>	0.1 ng DNA	Seed, fruits, chocolate, hazelnut creams, biscuits, cornflakes, snacks	[127]
		<i>Hsp1</i>	13 pg DNA, 100 ppm hazelnut in model pastry	Confectionery and bakery products	[128]
		<i>Cor a8</i>	10 ppm	Commercial food products and bakery products	[129]
		<i>ITS1</i>	0.1 ppm	Tree nuts, seeds, meat, plants, commercial food products	[90]

(continued)

Table 3
(continued)

Allergen	Method	Target gene	LOD	Food matrices tested	Reference
Cashew nut	RT-PCR	<i>2S albumin</i>	5 pg cashew DNA, 100 ppm cashew in chocolate cookies	Chocolate chip cookies, fruit and nut bars, cereal, crackers, chocolate, granola	[81]
		<i>2S albumin</i>	25 pg cashew DNA, 100 ppm cashew in model pastry	Tree nuts, cereal flours, cocoa and milk powder, chocolate, breakfast cereals, snacks, biscuits, nougate	[130]
		<i>Ana o3</i>	2 ppm	Pesto	[98]
Pecan	RT-PCR	<i>Vicilin-gene</i>	1 pg pecan DNA, 100 ppm pecan in model pastry	Pecan, walnut, cereals, flours, potato starch, biscuits, chocolate, praline	[82]
Walnut	PCR-RFLP	<i>MatK</i>	10 ppm walnut	Walnut-containing food products	[131]
	RT-PCR	<i>Jur r 2</i>	0.24 ng walnut DNA, 100 ppm walnut in pastry samples	Bakery, confectionary	[85]
Walnut	RT-PCR	<i>Vicilin-gene</i>	10 ppm of walnut	Commercial food products	[132]
		<i>Vicilin-gene</i>	NR	Confectionery and bakery products	[133]
		<i>Vicilin-gene</i>	NR	Confectionery and bakery products	[134]
Walnut/ Pecan	RT-PCR	<i>ITS1</i>	0.1 ppm	Tree nuts, seeds, meat, plants, commercial food products	[88]
Pistachio	PCR	<i>Rbel</i>	<100 mg/kg	Mortadella	[135]
	RT-PCR	<i>ITS</i>	4 ppm	Pastry	[136]
		<i>ITS</i>	0.1 ppm	Tree nuts, seeds, meat, plants, commercial food products	[87]
Macadamia	RT-PCR	<i>Vicilin-gene</i>	200 ppm	Confectionery and bakery products	[86]
Brazil nut	RT-PCR	<i>IIS Globulin</i>	1000 ppm	Commercial food products	[84]
		2S albumin	5 ppm	Processed food products	[137]
		2S albumin (<i>Bere1</i>)	2.5 ppm	Commercial food products	[100]

Sesame	RT-PCR	<i>2S albumin</i>	5 pg DNA, 50 mg/kg	Breakfast cereals, crackers, chocolate, cookies, fruit bars	[138]
		<i>Ses i 1</i>	50 pg DNA sesame, 500 ppm sesame in bread	Chocolate, sesame oil, tahini, snacks, bread	[139]
		<i>2S albumin</i>	50 ppm (w/w) in spice and flour	Spice, flour	[140]
Soy	PCR + nested PCR	<i>le1 (lectin gene)</i>	10,000 ppm protein concentrate	Processed meat products	[141]
		<i>trnL (chloroplast tRNA)</i>	NR	Soybeans	[117]
		Gly m Bd 30K	100 ppm soy flour	Legumes, cakes, hamburgers, sausage	[142]
		<i>SIRE-1</i>	10 ppm soy flour	Fish, bread, soymilk, tofu, lecithin, soy sauce, seasoning, biscuits	[143]
	RT-PCR	<i>le1</i>	2000 ppm soy flour, 10000 ppm soy protein isolate	Bread, flour	[144]
Soy/ Hazelnut	RT-PCR	<i>Lecitin</i>	100 ppm	Meat products	[145]
	RT-PCR	<i>Gly m Bd 28K/Cora1</i>	5–15 genome copies	Plant and tree nuts	[92]
Lupine	RT-PCR	<i>ITS1</i>	0.1 ppm	Legumes, seeds, nuts, spices, fruit, meat	[97]
Celery	PCR	<i>Manitol dehydrogenase gene</i>	490–1530 pg DNA, 1000 ppm celery in pat_e model	Meat pâte, seasoning, bouillon	[146]
	RT-PCR	<i>api g 1</i>	60 pg DNA (root), 30 pg (seed), 250 pg (powder)	Rinse solution after cleaning	[147]
		<i>Manitol dehydrogenase gene</i>	One DNA copy, 5–10 mg/kg celery seed	Sausage, seasoning, sauces, bouillon	[94]
		<i>Manitol dehydrogenase gene</i>	100–10 ppm celery powder	Meat, spice, flour, swab test	[140]

(continued)

Table 3
(continued)

Allergen	Method	Target gene	LOD	Food matrices tested	Reference
Mustard	RT-PCR	<i>sin a 1</i>	50 ppm in spice and flour	Spice, flour	[140]
	RT-PCR	<i>MADS D protein</i>	5 ppm	Meat products	[148]
Wheat	PCR +Nested PCR	<i>Triticin precursor</i>	500 ppm	Grains and processed food products	[149]
		<i>Wheat gliadin</i>	10–1000 ppm or 0.04–0.4 mg	Oat flake and kernels	[117]
		<i>Triticin precursor</i>	NR	Commercial processed foods	[150]
Wheat	RT-PCR	<i>Triticin precursor</i>	20 pg DNA/1.5 ppm	Commercial processed foods	[95]
Wheat and barley	RT-PCR	<i>w-Gliadin w-Secalin Hordein Avenin</i>	For four cereals, LOD; between 5 and 50 pg DNA or 0.1–100 ppm	Cereals, flours, bran, crunch, cookies, ice cream, pancakes	[151]
		<i>Acetyl-CoA carboxylase RALyase g -Hordein</i>	Wheat and barley: LOD 1 genome copy, LOQ 10 genome copies	Cereal flours and cereal-based food products	[152]
Wheat, barley and rye	RT-PCR	<i>TrmL (chloroplast rRNA)</i>	20 pg DNA (wheat), 2 pg DNA (barley, rye)	Cereals, bread, baby food	[153]
Buckwheat	RT-PCR	<i>ITS1 5.8S rRNA region</i>	1 ppm buckwheat (w/w) <10 ppm buckwheat flour	Buckwheat, wheat	[96]
Fish: Three mackerel species	PCR	5S rDNA NTS	NR	No food matrix; raw fish	[154]
Mackerel	PCR	<i>Parvalbumin</i>	5 ng mackerel DNA	Surimi, fish paste	[155]
Crustaceans: shrimp, lobster, crab, crawfish	PCR-RFLP	<i>16S rRNA gene</i>	15–20 ng DNA, <1000 ppm shrimp in raw pork meat	Raw pork, shrimp cake	[156]

<i>Multiplex</i>					
Wheat, buckwheat	Duplex PCR	Tricitin precursor, Allergenic storage protein	NR	Japanese food products	[102]
Peanut, hazelnut, celery, soy, egg, milk, almond and sesame	Tetraplex PCR	<i>Arah2</i> , <i>cora1 gene</i> , <i>Mid</i> , <i>Le1</i> , <i>2S albumin</i> , <i>Pru1</i>	100 ppm	Tree nuts, soybean, sesame, milk, eggs, commercial food products	[101]
Peanut and hazelnut	Duplex PCR	<i>Cor a1 Ara b2</i>	50 pg in standard solution	Breakfast cereals, muesli snack, wafer chocolates, biscuits	[110]
Sesame/ Hazelnut	Duplex RT-PCR	<i>Ses i 1/Cor a1</i>	10 pg DNA sesame and hazelnut, 50 ppm in cookies	Commercial food products	[157]
Celery/ White mustard	Duplex RT-PCR	<i>M6PR/MADS D protein</i>	50 ppm	Commercial food products	[158]
Pecan/Brazil nut	Duplex PCR	<i>Vicilin/Bere1</i>	1000 ppm	Commercial food products	[159]
<i>MLPA</i>					
Peanuts, cashews, pecans, pistachios, hazelnuts, sesame seeds, macadamia nuts, almonds, walnuts and brazil nuts.	LPA	<i>Arah2</i> , <i>anao3</i> , <i>Vicilin-gene</i> , <i>Bere1</i> , <i>Cor a gene</i> , CORA1.0401, 7S globulin mRNA, AMP2, extensin, sulfur-rich water-soluble seed protein	5 ppm	Tree nuts, soybean, sesame, milk, eggs, commercial food products	[104]

(continued)

Table 3
(continued)

Allergen	Method	Target gene	LOD	Food matrices tested	Reference
Sesame, soy, hazelnut, peanut, lupine, gluten, mustard, and celery.	LPA	<i>2S albumin, lectin (Le1), Cor a1, Arab2, ITS1, Glud1, SinA1, Apium graveolens</i>	10 ppm	Commercial food products	[105]
<i>Lab on a chip</i>					
Sesame/celery	Lab on a Chip	<i>ITS</i>	1 ppm sesame/50 ppm celery	Plants, meat balls, soups	[160]

PCR methodology is an *in vitro* enzymatic synthesis of specific DNA sequences, which relies on the ability of *Taq* polymerase, an enzyme that catalyzes DNA-copying, to remain stable at high temperatures, and two carefully selected oligonucleotides that serve as primers for the reaction and hybridize to opposite strands that flank the region of interest in the target DNA. PCR relies in a repetitive series of thermal cycles (heating and cooling) involving template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase. This results in the exponential accumulation of a specific fragment which termini are defined by the 5' ends of the primers. Thermal cycles are repeated 20–40 times. Because the primer extension products synthesized in one cycle can serve as a template in the next, the number of target DNA copies approximately doubles at every cycle amplifying the DNA of interest at a detectable level after 20–30 cycles.

The amplified product can be qualitatively visualized by staining after agarose gel electrophoresis, providing information on the size of the amplified product as is the case of the classical PCR reaction. The real-time PCR is a form of PCR where data are collected in “real time” as the reaction proceeds. Real-time PCR is a very sensitive technique, detecting down to a few molecules of target DNA. In contrast to classical PCR this method is based on fluorescence measurements to enable the visualization of PCR products during the amplified process. Real-time PCR has been the preferred approach for the quantitative analysis of specific DNA in a sample. In this technique, the reaction tube also contains a target-specific oligonucleotide probe together with a fluorescent reporter dye that has a quencher attached to it [80]. The detection of fluorescence is prevented by the proximity of the quencher to the dye. When the probe hybridizes to the amplified target DNA, the 5' exonuclease activity of the polymerase cleaves the probe, thereby separating the quencher from the dye, which is displaced by the newly synthesized DNA strand. The newly synthesized DNA strand becomes soluble and the fluorescence of the free reporter dye can then be measured. An increase in fluorescence is proportional to the amount of target DNA present in the sample.

The advantage of real-time PCR over traditional PCR is the ability to quantify target DNA content in the sample. It provides a higher degree of specificity given by a fluorescent probe, which anneal in the sequence flanked by the two primers. It also reduces interferences due to the formation of primer dimers and nonspecific products [81]. Also, real-time PCR is less prone to contamination and is suitable for automation. Numerous assays based on real-time PCR have been developed and have proved effective for the detection of a wide range of traces of food allergens in raw materials and processed foods [82–98]. López-Calleja et al. [89] reported a real-time PCR based on the *Arah2* gene and *ITS* region for the detec-

tion of peanut with a LOD of 10 and 0.1 ppm, respectively. These same authors described a real-time PCR for the detection of hazelnut based on the *ITS1* region, which reached a detection limit of 0.1 ppm [90]. Also, de la Cruz et al. [100] reported a PCR assay based on *2S albumin gene* (*Ber e1*) for the detection of Brazil nut in commercial food products, achieving a limit of detection of 2.5 ppm. In addition to the conventional singleplex, PCR or real-time PCR reactions have the potential of simultaneously amplifying more than one analyte in a food matrix by using a multiplex format. This multiplex approach was recently demonstrated by Koppel et al. [101], who developed two tetraplex real-time PCR. In one multiplex reaction they detected simultaneously peanut, hazelnut, celery, and soy and in another they detected egg, milk, almond, and sesame with a LOD of 100 ppm. Also a multiplex PCR assay was developed by Hashimoto et al. [102] for the simultaneous detection of wheat, buckwheat, and peanut. This multiplex format has the advantages of saving time, costs and reducing probability of cross-contamination. However, it often decreases sensitivity of the assay, compared to singleplex PCR.

Although PCR-based methods require more expensive equipment than immune-based techniques, they have some key advantages over immunochemical methods [80]. Food processing can affect the conformation and solubility of proteins. Harsh extraction methods cannot typically be used since they could further affect antibody-binding epitopes. With DNA however, harsher extraction buffers can be used without affecting the detection of the target DNA. Amounts of DNA also tend to be more stable than protein levels, which can vary between various species or varieties. An additional advantage is that PCR-based tests are available for detection of DNA from a number of allergenic sources for which ELISA methods may not be available.

2.2.2 MLPA, Multiplex Ligation-Dependent Probe Amplification

A ligation-dependent probe amplification (LPA) is another DNA-based assay where adjacent ligation probes bind to the target and are ligated, resulting in ligation products of specific size for specific target DNA. The 5' and 3' ends of the ligation product contain a common sequence used as targets for a common set of primers, used for a PCR amplification of the ligation product. Further detection of the amplicons is carried out by capillary electrophoresis. This technique has been used successfully for the screening of multiple products within the same reaction [99, 103]. Ehlert et al. [104] described a multiplex ligation-dependent probe amplification (MLPA) method for the detection of different tree nuts (peanut, cashew, pecan, pistachio, hazelnut, macadamia nut, almond, walnut, and Brazil nut) and sesame seeds with a LOD of 5 ppm. Also, Mustorp et al. [105] developed a quantitative 10-plex competitive MLPA method for the detection of eight allergenic ingredients (sesame, soy, hazelnut, peanut, lupin, gluten, mustard, and

celery) with a LOD of 10 ppm, depending on the allergenic ingredient. With allergen-MLPA, accurate quantitative results can be obtained. Although this DNA-based method does not detect the allergens as such, it shows many advantages, such as detecting a high number of targets simultaneously (multiplex format), providing quantitative specific, and sensitive results. Furthermore, this system is more flexible than real-time PCR, since it is modular and can therefore be easily adapted by removing or adding probe pairs for special purposes, as for example in those cases in which the number of targets (allergens) of interest increases.

2.2.3 DNA Biosensors

The detection of allergenic material in foods by using biosensor technology has been mainly limited to several antibody-based applications. However, biosensors have the potential to be developed also for DNA or PCR product detection. In this way, DNA-based biosensors, which are used for research rather than for routine analyses, are in increasing development. Sun et al. [106] developed an electrochemical DNA biosensor to detect peanut allergen Ara h1 in a peanut milk beverage with a LOD of 0.35–10–15 M. To this end, a 5'-SH and 3'-biotin dually labeled stem-loop probe was assembled on gold electrodes taking advantage of gold-thiol affinity. When the target was absent, the stem-probe was "closed," and after hybridizing with the target oligonucleotide, the conformation changed to "open." This change in stem-loop state modified the electron-transfer efficiency. Using a microfluidic-based platform (ImmuChip and ImmuSpeed, Diagnostics S.A.), Berti et al. [107] developed an enzyme-linked electrochemical genomagnetic assay to detect the presence of Cor a 1.04 allergen of hazelnut. The hybridization was performed on modified paramagnetic micro-beads via sandwich hybridization with a capture probe and a biotinylated signaling probe. Quantitative allergen detection was determined based on enzymatic kinetics obtained after trapping the enzyme labeled biotinylated hybrid in a multi-channel cartridge. Tortajada-Genaro et al. [108] developed a multiplexed DNA microarray method on a digital versatile disk (DVD) to simultaneously detect the presence of hazelnut, peanut, and soybean in foodstuffs. For that purpose, the surface of a DVD was printed with streptavidin and 5'-biotinylated probes that would hybridize with digoxin-labeled PCR products to be detected later on with a peroxidase-labeled digoxigenin antibody.

2.2.4 DNA Microarrays

The emerging micro technologies based on the use of microarrays have provided another possible tool for food allergen detection. A DNA Microarray also known as DNA chip or biochip is a very powerful tool for the simultaneous detection of multiple DNA sequences and multi-samples analysis. Microarrays based on oligonucleotides [109] or their analogues complementary to the DNA of several allergens have been developed [110], but few are commercially

available. The specific probes are immobilized on a solid surface by different techniques and recognize the complementary fluorescently labeled PCR amplicons. Probe-target hybridization is usually detected and quantified by the detection of fluorophore, silver-, or chemiluminescence-labeled targets to determine the nucleic acid sequences present in the target. The resulting fluorescent spots are further read with a fluorescence scanner at the proper wavelength. The method is qualitative and useful for a rapid screening.

2.2.5 Commercial DNA-Based Test Kits

The majority of kits commercially available for routine food allergen analysis rely on immunological methods, while the number of companies offering PCR/real-time PCR kits (*R-Biopharm*, *Alabdiagnostics*, *Biotecon*, *Innosieve*, *Kogene*, and *Gen-ial GmbH*) is rather limited, probably because its use for the detection of food allergens is yet not as widely accepted. However, whenever ELISA kits are not available or not specific for the analysis of a particular specific allergenic ingredient, PCR-based kits become the method of election. These commercial DNA-based methods include both PCR and real-time PCR, which are used as qualitative and semiquantitative tests respectively allowing in many cases multiplexed analysis. All the kits have LOD values around or below 10 ppm of the allergenic food in the sample, although this value is matrix dependent. The quantitative analysis of PCR-based kits depends on the availability of reference materials and on the knowledge of the genomic sequences. Standard materials are provided together with the commercially available kits or are produced “in house.” Among the different DNA-based commercial kits available at the moment, there are several real-time PCR kits provided by different suppliers for the detection of the following allergens: peanut, soy, lupine, almond, hazelnut, walnut, crustaceans, molluscs, sesame, celery, mustard, and gluten-containing cereals (wheat, rye, barley, and kamut), egg, fish, and milk (Table 4). However, up to date only one kit for peanuts provided by *Tepnel* has obtained the approval of full validated method by AOAC International. It should be noticed that the kits used for a collective group of allergens provide positive results across the different species belonging to the group. For example, the kit for fish is able to detect a large number of commercial fish species, and the kit for molluscs allows the detection of snails, mussels, and cephalopods. However, these kits are not designed to identify individual species.

Among the different DNA-based test kits, PCR kits are available in different formats, and real-time PCR kits are the most common. They generally contain an allergen reaction mix, an inhibition control (spike), a positive control, *Taq* polymerase and a fluorescence detection enhancer, which are sufficient for approximately 100 target DNA-specific reactions and 100 inhibition controls. The inhibition reactions help to minimize possible false-negative

Table 4
Commercial real-time PCR kits for detection of food allergens

Company name						
	R-Biopharm	4labdiagnostics	Biotecon diagnostics	Innosieve diagnostics	Kogene	Gen-ial GmbH
Targets	Celery	Celery	Celery	Celery	Crustaceans	Celery
	Crustaceans	Gluten	Gluten	Mustard	Soy	Soy
	Fish	Crustaceans	Soy	Soy	Milk	Lupine
	Gluten	Lupine	Peanut	Hazelnut	Peanut	Mustard
	Lupine	Molluscs	Hazelnut	Walnut	Egg	Sesame
	Molluscs	Mustard		Wheat		Almond
	Milk	Sesame		Barley		Cashew
	Mustard	Soy		Rye		Hazelnut
	Sesame	Peanut				
	Soy	Hazelnut				
	Peanut	Almond				
	Hazelnut	Pistachio				
	Macadamia	Cashew				
	Almond	Walnut				
		Pecan				

test results caused by inhibition of the PCR process. The qualitative analysis of sample DNA can be performed with a detection limit of ten DNA copies. There are also end point PCR kits, in which PCR products are visualized by agarose gel electrophoresis. These kits include PCR master mixes, requiring only the addition of template and *Taq* polymerase. The master mixes are generally already colored with a gel-loading dye so that a later additional procedural step is eliminated. Spike and control DNAs are also included to ensure the reliability of the tests. Last, are the PCR-ELISA test kits that allow the detection of specific DNA targets by means of the PCR. Once the target DNA is amplified, it is subsequently detected via a labeled hybridization probe in an ELISA-like technique. The kit contains a PCR-binding buffer for both the rehydration of streptavidin and binding of the PCR products; a buffer for the denaturation of the PCR products, an initial wash buffer for both the removal of the denaturation buffer and unbound PCR products; a buffer for the hybridization of the DNA probes; a stringency buffer for the removal of unbound DNA probes and for stringency testing of the hybridization; a conjugate buffer for the dilution of the concentrated antibody conjugate; a second wash buffer for the removal of unbound antibody conjugate; and finally a substrate solution for the colorimetric reaction and a stop solution to arrest the reaction. The sample analysis is qualitative with a detection limit of 10–20 DNA copies.

3 New Challenges and Future

Although many important key questions in the area of food allergy still remain unanswered such as: what defines a major allergen? Why some individuals develop food allergies and others do not? Which are the environmental factors? Could the environmental factors be monitored through epigenetics or modified by changes in the microbiome? Can tolerance to food be induced? Why some foods are more likely to trigger allergies than others? Does the route and timing of exposure have any role on the sensitization? Within the last years the area of food allergen and food allergy as a whole has witnessed some important developments. The techniques listed and discussed above on analytical methods for determination of allergens in food products are an important but minor branch of a major and complex picture. With the availability of a great number of genomic sequences and NMR/crystallographic structural studies, it is now possible to trace the taxonomy and genetic relationships of a great number of common allergens and their progenitor plant or animal cells. As a consequence, a large number of techniques directed to the diagnosis of the disease have been suggested, among them *in vitro* cell-based techniques such as basophil/mast cell degranulation, DC-T cell activation, and protein microarray techniques [16]. In particular, the demonstration that passive sensitization using human mast cell (LAD2) reproduced *in vitro* the clinical reactivity to peanut *in vivo* [111] is an important landmark and opens an important precedent where cumbersome Skin Prick Tests and food challenges can potentially be replaced by *in vitro* cellular tests [16].

With the recent advances in microbiome and our incremental understanding of gut physiology, the whole area of food tolerance induced by the commensal bacteria and their epigenetic implication as modulator of the immune system is also going through a major revolution. Initial animal studies on the effect of food allergy on the gut microbial ecology have demonstrated that disease-associated microbiota may play an important role in food allergy [112].

Regarding treatment, the last few years have witnessed interesting new ideas on oral, subcutaneous, and epicutaneous desensitization using a plethora of methods such as anti IgE antibodies (omalizumab), herbal therapy, chimeric allergen-IgG, Fcε blockage, lymph node injection, and patch therapy among others [16, 113]. The demonstration that resolution of cows's milk and egg allergy in children can be accelerated by increasing dietary baked allergen intake [114] is on line with recent and well-publicized successes in peanut Oral Immune therapy (OIT) treatments [115]. Maybe, the most compelling and interesting recent findings related to food allergens and the development of food allergy are the demonstration that early introduction of peanuts significantly decreased

the frequency of peanut allergy among high-risk children when compared with peanut avoidance at the age of 60 months [116]. Although these are still initial results and many aspects of the studies need to be worked out, these studies alter some preconceived ideas of our relationship with food allergens.

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